THE INITIATION OF GLYCOGEN BIOSYNTHESIS IN *ESCHERICHIA COLI*

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1. Introduction

Previous work from this laboratory has shown that a protein acts as the initial acceptor of glucose from UDPglucose for glycogen biosynthesis in rat liver [1,2].

Because of the different solubility of protein and glycogen in trichloroacetic acid, it has been possible to determine differentially the incorporation of radioactivity from labelled sugar nucleotide into protein or into glycogen.

Based on results obtained with rat liver preparations [3], we have proposed the participation of two enzymes and an acceptor protein in the initiation of glycogen biosynthesis.

We have applied the same approach to the study of glycogen initiation in bacteria. In this paper we report the isolation from *Escherichia coli* of an enzyme complex which synthesizes a 1,4-α-glucoprotein not only from ADPglucose, which is considered to be the specific donor in bacterial glycogen biosynthesis but also from UDPglucose.

2. Materials and methods

Unlabelled UDPglucose, ADPglucose and ADP-[^{14}C] glucose (228 mCi/mM) were purchased from Sigma Chemical Co.

UDP-[^{14}C] glucose (309 mCi/mM) was prepared according to the method of Wright and Robbins [4] with minor modifications.

The incubations with degradative enzymes were as follows: pronase (Calbiochem), β-amylase (Mann) and salivary α-amylase were used as previously described [1]. Degradation with *Rhizopus* amyloglucosidase (obtained from Sigma) was according to Lee and Whelan [5] at 55°C.

Paper chromatography of hexoses and oligosaccharides was as previously described [3].

2.1. *Enzyme preparation*

E. coli AT_9 (met$^-$, RNAse 1$^-$) was used throughout these studies. The cells were grown in B-tryptone salts medium [6] with the addition of 0.2 g/l of $MgSO_4$ and 1.0 g/l of NH_4Cl.

The cells from 2 litres of culture in the early exponential phase were collected by centrifugation. After washing twice with 10 mM glycine–NaOH buffer (pH 8.7) containing 10 mM NaEDTA, they were suspended in 5 ml of 50 mM Tris–HCl (pH 8.4) containing 0.35 M sucrose. Spheroplasts were obtained by incubation with lysozyme (100 μg/ml) in the presence of 0.8 mM EDTA (pH 8.4) as described by Viñuela et al. [7]. 'Protoplast' formation was complete after approximately 60 min at room temperature and lysis then occurred on adding 45 ml of a 5 mM $MgCl_2$ solution. Deoxyribonuclease (Sigma) (250 μg) was added and incubation was continued for a further 30 min period at 37°C. All subsequent steps were performed at 4°C. The lysate was centrifuged at 7700 g for 20 min. After discarding the sediment, the supernatant was centrifuged at 105 000 g for 150 min. The pellet thus obtained was suspended in 10 mM glycine–NaOH buffer (pH 8.7) containing 5 mM NaEDTA and 0.25 M sucrose solution (about 10 mg/ml protein) and used as a source of enzyme.

2.2. *Assay procedure for glucoprotein formation*

The standard incubation mixtures contained 100 mM glycine-NaOH (pH 8.7) buffer; 5 mM dithiothreitol;

100 mM NaEDTA; 1 mM UDP-[^{14}C] glucose or ADP-[^{14}C] glucose (50 000 cpm) and 10 μl of enzyme, in a final vol of 100 μl. The mixture was incubated at 37°C for the times indicated.

2.3. *Measurement of glucoprotein formation*

Two methods were used: the 'trichloroacetic acid precipitation method' already described [1] or the 'acid-treated filter paper method' which consists in introducing directly into the test tube a roll of acid-treated filter paper [8].

When the standard reaction mixture was supplemented with 1 mg of glycogen there was no glucoprotein formation as occurs with the liver enzyme [1]. Therefore the assay used to measure the [^{14}C] glucose incorporated into glycogen was the 'KOH method'. This procedure was as follows: the reaction mixture was digested with 33% KOH, glycogen precipitated twice with ethanol, dissolved in water and counted with Bray's solution [9].

3. Results and discussion

The bacterial glycogen synthases previously described [10–13] synthesize a 1,4-type α-glucan from ADP-glucose only. This occurs either in the absence or in the presence of primer.

As shown in table 1, our *E. coli* enzyme preparation, which is almost devoid of glycogen, catalyzes the transfer of glucose from ADP-[^{14}C] glucose as well as from UDP-[^{14}C] glucose into a protein acceptor, forming a trichloroacetic acid insoluble compound. This occurs in the absence of primer.

On the other hand, when glycogen was added to the

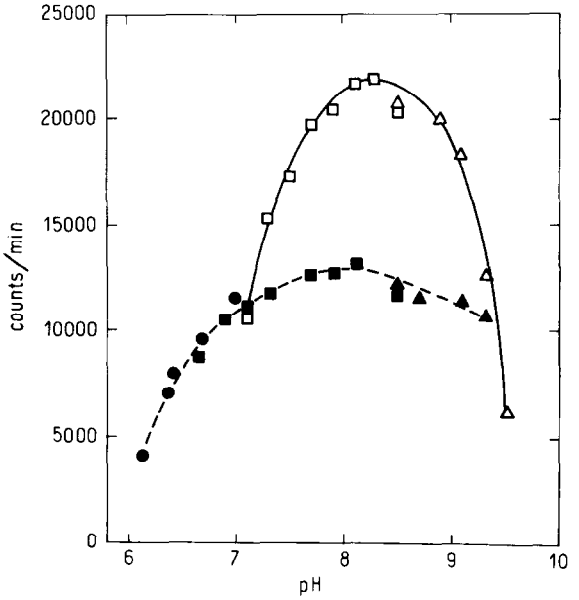

Fig.1. pH versus activity curve for synthesis is glucoprotein. The activity detected with UDP-[^{14}C] glucose (——) and ADP-[^{14}C] glucose (– – –) was measured as in the standard incubation mixture but with the following buffer solutions at different pH values: (□, ■) glycylglycine; (△, ▲) glycine-NaOH; (●) imidazole–HCl. The assay method used was that of 'acid-treated filter paper' [8].

Table 1
Sugar nucleotide specificity in glucoprotein synthesis

Reaction mixture	Labelled nucleotide	^{14}C Incorporation into Protein (μmol in 30 min)	Glycogen
Complete	UDP-[^{14}C] glucose	0.017	<0.0001
Complete + glycogen (1 mg)	UDP-[^{14}C] glucose	<0.0001	0.002
Complete	ADP-[^{14}C] glucose	0.009	<0.0001
Complete + glycogen (1 mg)	ADP-[^{14}C] glucose	<0.0001	0.025

Conditions were as described for the standard incubation mixture. The incubation time was 30 min. The assay methods used were that of "acid-treated filter paper" [8] for incorporation into protein and 'KOH' for incorporation into glycogen [9].

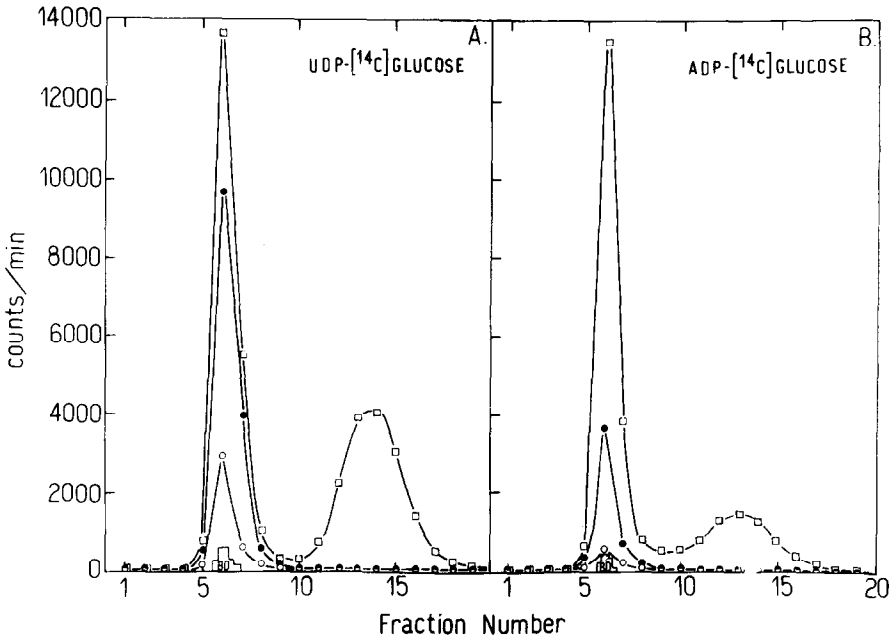

Fig.2. Sephadex G-50 chromatography of glucoprotein. The incubation mixture was as described under Materials and methods except that the amount of enzyme was increased to 0.4 mg, and 2×10^6 cpm of the labelled sugar were present (UDP-[^{14}C]-glucose: 309 μCi/μmol: ADP-[^{14}C]glucose: 228 μCi/μmol). After 60 min at 37°C the complete reaction mixture was applied to a Sephadex G-50 column (1 × 18 cm) and eluted with 10 mM glycine–NaOH (pH 8.7) buffer containing 10 mM EDTA at 4°C. Fractions of 0.5 ml each were collected. Radioactivity was determined by taking a 20 μl aliquot and counting directly with Bray's solution (□) or by placing 20 μl on 'acid-treated filter paper' (60 min (●) or 5 hr (○) after emerging from the column) and measuring the trichloroacetic acid precipitable fraction by the method already described [8]. BD: blue dextran.

incubation mixture there was no glucoprotein formation; instead, UDP-[^{14}C]glucose gave rise to a small incorporation into glycogen, while ADP-[^{14}C]glucose, which is the preferred glucose donor in bacteria, gave rise to a 10-times greater incorporation into glycogen. When GDP-[^{14}C]glucose was used, no incorporation at all was detected.

These results are representative of the behaviour encountered in a survey of several enzymatic preparations. Sometimes the amount of glucose incorporated from UDP-[^{14}C]glucose into the trichloroacetic acid precipitable compound was up to four times higher than that from ADPglucose; while in others it was equal to or lower. This might be related to the content of endogenous primer in the different preparations.

As shown in fig.1, maximal activity for incorporation into the trichloroacetic acid insoluble fraction with UDP-[^{14}C]glucose as donor was at pH 8.3. The curve obtained with ADPglucose shows that the enzyme is almost fully active over a rather broad pH range, from 6.0 to 9.0.

The radioactive products obtained after incubation in the presence of UDPglucose or ADPglucose were submitted to gel filtration on Sephadex G-50. As shown in fig.2, a radioactive product eluted with the void volume. This suggested that the radioactivity was associated with a macromolecule. The characterization of this macromolecule was performed as indicated in table 2. It was shown that the product is sensitive to pronase and a variety of glucosidases, indicating that a 1,4-α-glucanprotein is formed.

Some evidence was obtained indicating that this radioactive product was partially degraded during incubation. In fact the trichloroacetic acid precipitable radioactivity found in the void volume (fig.2) decreases 70% after storage at 0°C for 5 hr. After this period the presence of labelled oligosaccharides was detected (fig.3). This is consistent with the hypothesis that an

Table 2
Effect of enzymes on release of the radioactivity from glucoprotein

Enzyme treatment	Remaining cpm in the trichloroacetic acid precipitable product from	
	UDP-[^{14}C]glucose	ADP-[^{14}C]glucose
None	20 800	14 310
Pronase	1849	1300
α-Amylase	1033	1479
β-Amylase	1068	586
α-Glucosidase	103	93

A scaled-up standard reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 10% cold trichloroacetic acid. The trichloroacetic acid precipitate was washed with butanol and dried before use. The dried protein precipitate was incubated with the degradative enzymes under the conditions described in Materials and methods. After incubation, cold 5% trichloroacetic acid containing LiCl (2 mg/ml) was added and the radioactivity in the pellet and in the supernatant fluid was measured.

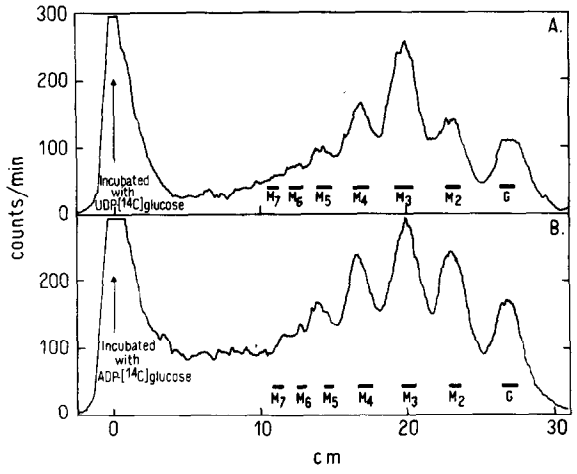

Fig.3. Distribution of radioactivity following paper chromatography of the material containing the radioactive glucoproteins eluted in the void volume from the Sephadex G-50 column (fig.2). After 5 hr at 4°C an aliquot of 40 μl from fraction number 6 from each experiment in fig.2 was spotted on Whatman N°1 paper for chromatography in butanol–pyridine–water (4:3:4). A mixture of malto-oligosaccharides [3] was used as internal standard. The paper strips were scanned for radioactivity with a Packard Radiochromatogram Scanner and afterwards revealed with alkaline silver nitrate. G: glucose; M_2, maltose; M_3, maltotriose and so on.

endogenous amylase-type activity is able to degrade the oligosaccharide chain bound to the protein acceptor. These oligosaccharides co-chromatographed with oligosaccharides of the maltose series and are degraded by β-amylase and α-glucosidase to maltose and glucose. This is additional evidence that the sugar–sugar linkage formed on transfer from both sugar nucleotides to the protein acceptor is of the α-1,4 type.

Further evidence for the existence of the amylolytic activity was obtained by incubation of radioactive glycogen eith the enzyme preparation. The results are shown in table 3. The addition of unlabelled ADP-glucose stimulates the amylolytic activity; this might be due to lengthening of the chains of the glycogen molecule.

The presence of an endogenous amylolytic activity could explain the results obtained by Fox et al. [11]. They have reported that although initially almost all of the alcohol insoluble product was also insoluble in 10% trichloroacetic acid, after a certain incubation time the trichloroacetic acid insoluble compound reached a maximum which was about 60% of the amount of the alcohol insoluble product.

On the basis of our results, we suggest that the *E. coli* enzyme complex could initiate glycogen synthesis by forming a glucoprotein with 1,4-α-glucosidic linkages, not only from ADPglucose, but also from

Table 3
Distribution of radioactivity in paper chromatogram of oligosaccharides obtained from incubating labelled glycogen with the *E. coli* enzyme

Reaction mixture	Percentage of radioactivity in	
	Origin	Oligosaccharide zone from maltoheptaose up to glucose
Minus enzyme	100	0
Complete	44	56
Complete plus 0.1 μmol of unlabelled ADPglucose	16	84

The complete reaction mixture in a total volume of 100 μl contained 5 mM dithiothreitol; 100 mM glycine-NaOH (pH 8.7) buffer; enzyme and [^{14}C]-glycogen (0.1 mg and 13 670 cpm). The labelled glycogen was prepared as previously described [8]. After 3 hr at 37°C the mixture was freed from salts by passage through an Amberlite MB-3 resin column. The eluates were concentrated and spotted for paper chromatography in butanol-pyridine-water (4:3:4). In order to scan quantitatively for radioactivity, the paper strips were cut into 1 × 2.5 cm rectangles and counted in a scintillation spectrometer after adding 3.5 ml of toluene-based scintillation fluid.

UDPglucose. The elongation of the glycogen chains could only occur by transfer from ADPglucose. That is to say, glucose is transferred to protein or to oligosaccharide chains covalently linked to the protein, from both sugar nucleotides. Once the oligosaccharide acquires a certain chain length, only ADPglucose would function as efficient glucose donor.

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